

Reproductive alterations in adult grass shrimp, *Palaemonetes pugio*, following sublethal, chronic endosulfan exposure

E.F. Wirth *, S.A. Lund, M.H. Fulton, G.I. Scott

Center for Coastal Environmental Health and Biomolecular Research, National Ocean Service, NOAA, 219 Fort Johnson Road, Charleston, SC 29412, USA

Received 16 February 2001; received in revised form 14 August 2001; accepted 14 August 2001

Abstract

Grass shrimp (*Palaemonetes pugio*) populations exposed to anthropogenic contaminant sources in South Carolina (SC) have reduced densities when compared with populations at SC-reference sites. This laboratory study examined the effects of a commonly used agricultural insecticide, endosulfan, on grass shrimp reproduction. Reproductively active grass shrimp were chronically exposed to sublethal concentrations of endosulfan (200 or 400 ng/l) for 43 days. The cumulative number of females that became gravid and the rate at which they became gravid were measured. Endosulfan exposure reduced the cumulative number of gravid females by 31% in the 200 ng/l exposure and 39% in the 400 ng/l exposure. The first appearance of gravid females in the population was significantly delayed in treated populations compared with the control treatment in a dose dependent manner. Clutch size in these gravid females was not significantly different among the treatments. Additionally, there was no difference in the onset of reproduction in the treated populations. These results implicate a population reduction due to a decrease in the overall number of females becoming gravid in a population over time, not a reduction in clutch size per individual. While the mechanisms of action have yet to be defined, these results indicate that sublethal endosulfan concentrations may have a negative effect on grass shrimp reproductive biology. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reproduction; Endosulfan; Grass shrimp

1. Introduction

Reduced densities have been reported in grass shrimp populations exposed to nonpoint source agricultural runoff and those living in highly disturbed habitats in urbanized estuaries (Finley et al., 1999; Scott et al., 1999). Two high salinity estuaries with tidally driven flow, but different

land uses, have shown differences in grass shrimp population densities (Finley et al., 1999; Scott et al., 2000). Over several years, the Murrells Inlet, SC (MI) estuary (urban land use) has consistently produced lower population densities when compared with the undeveloped North Inlet (NI) estuary. Between 1990 and 1998, annual MI shrimp population densities have been reduced by 80–90%. Within the MI estuary, there also appears to be a gradient of impact associated with the level of urbanization. In the southern region of MI

* Corresponding author.

which has larger undeveloped tracts of land (i.e. Hunting Island State Park), land use more closely resembles the NI estuary, and in turn, population densities are not as severely impacted. This trend is also consistent with the contaminant profiles of xenobiotics (Finley et al., 1999). Evaluations of the life histories of these two populations indicated that females within the MI estuary have slightly longer maturation periods (the time from larvae to gravid female); resulting in more females being present during the winter months, which may lead to altered sex ratios (Finley et al., 1999). Similar population trends were evident in tidal creeks that received agricultural nonpoint source runoff (endosulfan, fenvalerate, and azinphos-methyl) along the South Carolina coast (Scott et al., 1999).

While important inferences can be made from these field assessments, laboratory tests linking reproduction effects in crustaceans to sublethal pesticide exposure are limited mainly to small crustaceans such as *Daphnia magna* (Zou and Fingerman, 1997) or the harpacticoid copepods (Wirth et al., 1998; Chandler and Green, 1996). To date, only one other study has investigated reproductive impacts associated with contaminant exposure using the grass shrimp *Palaemonetes pugio* (Oberdörster et al., 2000). This species has also been utilized in acute toxicity testing (Schimmel et al., 1977; Buikema et al., 1980; Wirth et al., 2001) and as an indicator of sublethal effects (Key et al., 1988; Key and Fulton, 1993). However, mechanistic links between exposure and reproduction have not been studied.

In light of recent reports that endosulfan may alter endocrine function (Soto et al., 1994; Colborn et al., 1993), consideration of the pathways by which this compound might affect reproduction in invertebrates is warranted. In terms of viable endocrine endpoints, the United States Environmental Protection Agency (US EPA) defines three areas of emphasis: growth, reproduction, and development. Furthermore, the US EPA suggests that research should focus “primarily on development of reproductive capability” (Kavlock et al., 1996). In terms of the evaluation of endocrine-associated endpoints, such as reproduction in invertebrates, it is important to remember

that our understanding of the reproductive physiology of crustaceans is small in comparison to mammals and insects (commonly used as the model for marine invertebrates). To this end, ecological risk assessments utilizing invertebrates to evaluate endocrine disruption will depend on reproductive and developmental assays (Hutchinson et al., 2000).

Endosulfan has been shown to be an endocrine disrupting chemical in cell lines transfected with estrogen receptors. Soto et al. (1994) reported that the estrogen-sensitive human breast cell line MCF7 was responsive to endosulfan exposure at levels similar to that of DDT, but endosulfan did not act as efficiently as the natural hormone estradiol. Additional testing with *Daphnia magna* indicated that endosulfan did not interfere with sexual differentiation but did inhibit molting (Zou and Fingerman, 1997). In terms of reproductive output, there have been no published reports examining the effects of endosulfan exposure on crustaceans at environmentally relevant concentrations.

It is unclear whether contamination per se causes an acute decline (mortality) in grass shrimp populations in the environment or if there is a chronic sublethal response within the population, such as an alteration in the reproduction of adults. Before that question can be answered, laboratory studies linking exposure to reproductive declines must be undertaken. The objectives of this study were to investigate the reproductive biology of grass shrimp exposed to sublethal concentrations of endosulfan and to determine if sublethal, chronic contaminant exposure could account for population level changes.

2. Materials and methods

Gravid *Palaemonetes pugio* were collected from Leadenwah Creek, Wadmalaw Island, SC and transported to the laboratory. They were acclimated to room temperature (18–23 °C) and 20‰ salinity. Individual gravid shrimp were caged in 2 × 2 × 3 cm compartments with 1 mm Nitex™ walls, which allowed for newly hatched larvae to escape predation from adult shrimp. Upon hatch-

ing, larvae were collected and reared in 70 l aquariums until they reached maturity (> 5 mm, Knowlton and Williams, 1970). Newly hatched larvae were initially fed 24-h-old hatched brine shrimp (*Artemia* spp.) twice daily. Tetramin™ flake food was added to the diet as the shrimp grew, eventually leading to a diet of flakes twice daily and ~ 2 ml of concentrated, 24-h-old *Artemia* spp. every other day. This laboratory-hatched population was cultured for ~ 6 months before testing. Reproductively active females were evident in all cultured populations prior to testing. Approximately 10 weeks prior to exposure, the cultured shrimp were separated and isolated in sex-specific aquariums. This allowed all females that were exhibiting ripe ovaries or egg clutches to exhaust their current spermatophore.

The chronic exposure chambers (control and endosulfan) consisted of 70-l aquariums containing 20‰ seawater, and biological filtration systems driven by an Aquaclear 300 filter. The filter media [Eheim Filter Floss (Eheim, Germany) and crushed oyster shell] were selected to minimize pesticide absorption, which would normally occur on activated carbon, while providing for an effective bacterial and particulate filter.

The treatment tanks (acetone only, 200 and 400 ng endosulfan per l) were pre-dosed for three weeks prior to the inclusion of shrimp, in order to bind any active sites and create a constant exposure system. All treatments and the controls received the same acetone concentration (0.01%). This acetone concentration is below the NOEC of 0.1% reported by Mayer (1987). Technical grade endosulfan was dissolved in acetone to create a stock of 10 mg/l in acetone. This stock endosulfan solution was used to dose the treatment tanks daily at a nominal concentration of 200 or 400 ng/l. The low endosulfan dose was based on an LC_{15} for male grass shrimp (Wirth et al., 2001) and the high dose was approximately twice the LC_{15} . Water quality parameters [salinity (20‰), temperature (24 °C), dissolved oxygen ($> 70\%$), and pH (7.8–8.2)] were measured daily in the control tanks. There were two replicate tanks per treatment. Fifty ml water samples from each treatment were analyzed for endosulfan concentrations weekly according to the methods of Scott

et al. (1992). These samples were immediately extracted onto C-18 solid phase extraction cartridges and eluted with 12 ml of pesticide-grade ethyl acetate. Ten μ l of δ -HCH were added as an internal standard for quantification. The extracts were solvent exchanged into *iso*-octane under nitrogen and reduced to 1 ml for analysis by GC-ECD.

After the acclimation period, 48 cultured females and 18 cultured males were added to each treatment tank. Tanks were examined each morning for females with ripe ovaries and newly extruded egg clutches. When a gravid female (holding a clutch under the abdomen) was identified, the individual was removed without replacement. Observed mortalities were removed and recorded. The first day that any gravid female was observed was designated as time zero (T_0) for the experiment. Weight and length were recorded and a unique identifying number was assigned to each gravid female. The individual was gently dried, wrapped in solvent rinsed aluminum foil and flash frozen in liquid nitrogen. The frozen samples were placed in containers and stored at -80 °C. The experiment was terminated on day 27, when one control replicate was observed to have unusually high mortality compared with the previous observations. A clutch size index (no. of eggs per mm female) was determined for gravid females by gently teasing the clutch from the abdomen and counting all embryos, then dividing the clutch size by the total length of the female. There was an $n = 4$ for the Control and 200 ng/l treatment and an $n = 3$ for the 400 ng/l treatment.

These reproductive data were compiled and analyzed for differences in total number of gravid females and the rate at which the populations produced gravid females. Linear regression estimates for each treatment were calculated using a regression of time versus the cumulative number of gravid females. The slope of each line was used as an estimate of the rate at which females became gravid during the exposure. Using the methods from Zar (1999) to compare multiple linear equations (slope and y -intercept), statistical differences among treatments were assessed. Clutch size was normalized for female length and compared using a one-way analysis of variance (ANOVA) ($\alpha = 0.05$).

3. Results

Average water concentration for the endosulfan treatments was 134.4 ng/l (200 ng/l nominal) and 249.1 ng/l (400 ng/l nominal) over the course of the exposure period. Endosulfan was not detected in the control aquariums (DL = 1 ng/50 ml). Water quality parameters from the tanks averaged (\pm S.D.) 20.4‰ (\pm 0.8), 24.3 °C (\pm 0.4), 8.0 pH (\pm 0.1), and 6.6 mg/l (\pm 0.5) for salinity, temperature, pH and dissolved oxygen, respectively.

Average mortality was quite low and consistent among the replicates. The estimated treatment survival ranged from 94 to 100% among all replicates. At the end of the exposure, there was a 31% decrease in the number of gravid in the 200 ng/l exposure when compared with the controls and a

39% decrease was observed in the 400 ng/l treatment. Fig. 1 depicts the rate at which the treatment populations produced gravid females over the course of the study. Statistically, the calculated rate at which gravid females were produced was lower when compared with the control (Table 1).

There was no difference in the clutch size index among the treatments (Table 2). Average clutch size per mm for each randomly selected female was 2.123 (\pm 0.690) embryos per mm in the control, 1.968 (\pm 0.555) embryos for the 200 ng/l treatment and 2.397 (\pm 0.122) embryos for 400 ng/l treatment. Additionally, there was not a difference in female length (range 26–31 mm). Means were calculated from a sample size of 4 for Control and 200 ng/l and a sample size of 3 for the 400 ng/l treatment.

Occurrence of Gravid Females during Endosulfan Exposure

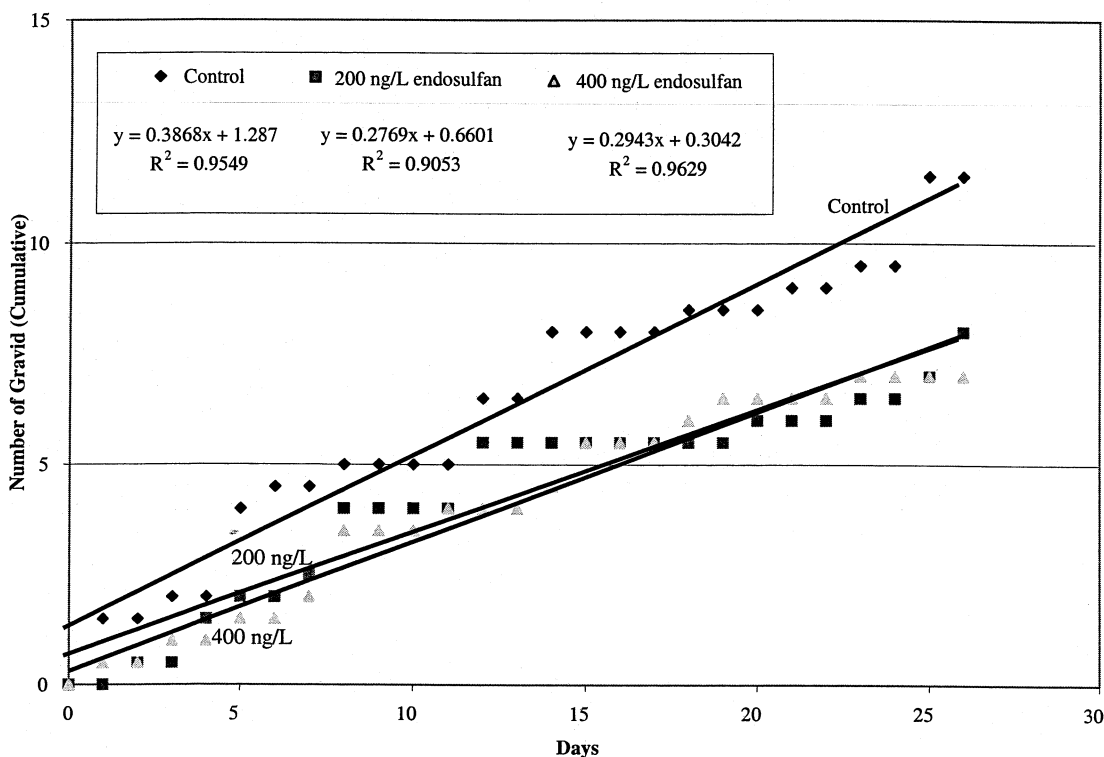


Fig. 1. The cumulative number of gravid females during 6 weeks of exposure to endosulfan. Linear regression estimates of each curve were determined (MS Excel97) and used to estimate the rate of reproduction within each treatment.

Table 1

Following Zar (1999), the three reproductive trendlines were compared based first on the slope of the line and then if needed the y -intercept

| Comparisons | Control | 200 ng/l | 400 ng/l |
|-------------|------------------------|--------------------------------|-----------------------|
| Control | | Slopes not equal* | Slopes not equal* |
| 200 ng/l | No y -intercept Test | | Slope not significant |
| 400 ng/l | No y -intercept Test | y -intercept not significant | |

Significantly different slopes (*) were observed and the Tukey's Studentized Range Test for Slopes indicated that the endosulfan treatments were significantly different from the control. Thus, no y -intercept test was needed to differentiate between the control and the two treatments. The slopes were not different between the 200 and 400 ng/l endosulfan exposures, and a test of elevation (y -intercept) was performed but these data sets were not found to be significantly different.

4. Discussion

Scott et al. (1992) noted significant reductions in grass shrimp densities, as well as decreases in higher trophic levels (mummichogs and blue crabs), at sites chronically polluted with endosulfan in South Carolina estuaries. Factors contributing to the decline of these populations such as acute mortality, chronic effects, decreases in egg production or recruitment have not been definitively identified, but Sikora (1977) indicated that peak grass shrimp densities in SC estuaries occur in the fall (November). Based on the data reported here, a reduced spring reproductive event would produce fewer offspring capable of producing the fall peak. Additionally, if developmental times are increased, the November peak could be missed altogether.

Changes in population recruitment patterns (e.g. numbers, sex ratios, or time of first-of-the-year reproduction) could be related to the sublethal contaminant effects. Toxicity based on endosulfan causes paralysis by blocking neurotransmitter synapses, however, sublethal effects on the nervous system may alter behavior or basal metabolism, affecting the direct, sustained shrimp to shrimp contact needed for mating (Berg and Sandifer, 1984). Generally, daily observations did not indicate any differences in behavior; however, no quantitative observations regarding behavior were made.

Other crustacean researchers have reported reproductive trends associated with contaminant exposure. Lutofo and Fleeger (1997), Wirth et al. (1998) have reported decreases in the number of

gravid females after PAH-contaminant exposure. While neither of these studies reported significant differences between control and PAH treatments, there was a dose dependent decrease in the number of gravid females at the end of each experiment. Lutofo and Fleeger (1997) exposed the benthic copepod *Schizopera knabeni* to a maximal phenanthrene concentration of 217 $\mu\text{g/g}$. As concentrations increased, the percent of the test community that was ovigerous dropped from ~ 30 to 5%. When the copepod *Amphiascus tenuiremis*

Table 2

Clutch size determination from each treatment

| Sample no. | No. embryos per mm gravid female | | |
|--------------------------------|----------------------------------|------------|------------|
| | Control | 200 ng/l | 400 ng/l |
| 1 | 2.153 | 1.966 | 2.538 |
| 2 | 2.519 | 2.033 | 2.333 |
| 3 | 2.679 | 2.615 | 2.321 |
| 4 | 1.143 | 1.259 | na |
| Average | 2.123 | 1.968 | 2.398 |
| S.D. | 0.689 | 0.555 | 0.122 |
| Average clutch size | 57.75 | 55.00 | 64.67 |
| Number of gravid females | 12 | 8 | 7 |
| Potential Young (% of control) | 693 (na) | 440 (62.5) | 452 (65.2) |

Gravid females were randomly selected and their clutches were counted. The clutch was then normalized to the female length, resulting in a clutch index (No. embryos per mm female length). There were no significant differences calculated between the controls and endosulfan treatments using a one-way ANOVA on Ranks.

was exposed to a PAH mixture at concentrations ranging between 1 and 10 µg/g, Wirth et al. (1998) reported a >30% decrease in the number of gravid females. The PAH results compare favorably to preliminary endosulfan exposures where there was a 26% reduction in the number of gravid females reported (Wirth, 1999) and the 30–40% reduction reported in this study. Scott et al. (2000) reported that the grass shrimp populations at a site impacted by chronic insecticide runoff alone (no acute toxicity and includes endosulfan) were reduced by nearly 35%, which is very similar to the reduction reported in the number of gravid females occurring in this study. Additionally, when *D. magna* were exposed to endosulfan at levels between 0.2 and 0.5 the reported LC50 (0.2–0.31 mg/l), the average number of young per female, number of broods per female and the average number of days to first reproduction were significantly different from controls (Fernandez-Casalderrey et al., 1993). In a subsequent study, the same researchers reported a decrease in feeding rate and filtration at the same concentrations (Fernandez-Casalderrey et al., 1994).

In summary, chronic endosulfan exposure negatively affected reproduction in terms of the rate at which females grass shrimp became gravid. The effect of this type of delay on a population may not be evident in other regulatory bioassays. There were no effects on adult survival, clutch size, or hatching success. However, the potential offspring in treated populations was calculated to be 63–65% of the control population. This reproductive effect could potentially lead to less developed, smaller and fewer offspring being available to the ecosystem during seasons where food availability is reduced (winter). This in turn could increase predation pressures on the population, with no immediate source of recruitment from the adult population. This biphasic population phenomenon in endosulfan-exposed shrimp populations (i.e. reduced reproductive output and increased predation pressure) needs to be further examined. Future research addressing potential mechanistic relationships between reproduction and endosulfan levels, lifestage sensitivities, and molting frequency also needs to be evaluated.

Acknowledgements

A portion of this research has been supported through collaboration with the University of South Carolina and the US EPA through grant number USEPA R-827397-01. The research described in this paper has been funded wholly or in part by the US EPA, but it has not been subjected to the Agency's required peer and policy review and, therefore, does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

References

- Berg, A.V., Sandifer, P.A., 1984. Mating behavior of the grass shrimp *Palaemonetes pugio* Holthuis (Decapoda, Caridea). *J. Crust. Biol.* 4, 417–424.
- Buikema, A. Jr, Niederlehner, B., Cairns, J. Jr, 1980. Use of grass shrimp in toxicity tests. In: Buikema, A. Jr, Cairns, J. Jr (Eds.), *Aquatic Invertebrate Bioassays*. American Society for testing and Materials, USA, pp. 155–173 ASTM STP 715.
- Chandler, G.T., Green, A.S., 1996. A 14-day harpacticoid copepod reproduction bioassay for laboratory and field contaminated muddy sediments. In: Ostrander, G. (Ed.), *New Techniques in Aquatic Toxicology*. Lewis Publishers Inc, pp. 23–39.
- Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 101 (5), 378–384.
- Fernandez-Casalderrey, A., Ferrando, M.D., Andreu-Moliner, E., 1993. Effects of endosulfan on survival, growth and reproduction of *Daphnia magna*. *Comp. Biochem. Physiol.* 106C (2), 437–441.
- Fernandez-Casalderrey, A., Ferrando, M.D., Andreu-Moliner, E., 1994. Effect of sublethal concentrations of pesticides on the feeding behavior of *Daphnia magna*. *Ecotox. Environ. Safety* 27, 82–89.
- Finley, D.B., Scott, G.I., Daugomah, J.W., Layman, S.L., Reed, L.A., Sanders, M., Sivertsen, S.K., Strozier, E.D., 1999. An ecotoxicological assessment of urban and agricultural nonpoint source runoff effects on the grass shrimp, *Palaemonetes pugio*. In: Lewis, M.A., Powell, R.L., Nelson, M.K., Henry, M.G., Klaine, S.J., Dickson, G.W., Mayer, F.L. (Eds.), *Ecological Risk Assessment for Wetlands*. SETAC Press, Pensacola, FL, pp. 243–273.
- Hutchinson, T.H., Brown, R., Brugger, K.E., Campbell, P.M., Holt, M., Lange, R., McCahon, P., Tattersfield, L.J., van Egmond, R., 2000. Ecological risk assessment on endocrine disruptors. *Environ. Health Perspect.* 108 (11), 1007–1014.
- Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac,

- M.J., Maczka, C., Miller, R., Moore, J., Ropland, R., Scott, G., Sheenen, D.M., Sinks, T., Tilton, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disrupters: a report of the US EPA-sponsored workshop. *Environ. Health Perspect.* 104 (Suppl. 4), 715–7410.
- Key, P.B., Fulton, M.H., Layman, S.L., Scott, G.I., 1988. Azinposmethyl exposure to grass shrimp (*Palaemonetes pugio*) life stages with emphasis on larval acetylcholinesterase activity. *Bull. Environ. Contam. Toxicol.* 60, 645–650.
- Key, P.B., Fulton, M.H., 1993. Lethal and sublethal effects of chlorpyrifos exposure on adult and larval stages of the grass shrimp, *Palaemonetes pugio*. *J. Environ. Sci. Health B28* (5), 621–640.
- Knowlton, R.E., Williams, A.B., 1970. The life history of *Palaemonetes vulgaris* (Say) and *P. pugio* Holthuis in coastal North Carolina. *J. Elisha Mitchell Sci. Soc.* 86, 185.
- Lutofo, G.R., Fleeger, J.W., 1997. Effects of sediment-associated phenanthrene on survival, development and reproduction of two species of meiobenthic copepods. *Mar. Ecol. Prog. Ser.* 151, 91–102.
- Mayer, F., 1987. Acute toxicity handbook of chemicals to estuarine organisms. EPA/600/8-87/017.
- Oberdörster, E., Brouwer, M., Hoexum-Brouwer, T., 2000. Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males and offspring of exposed females. *Environ. Health Perspect.* 108 (7), 641–646.
- Schimmel, S.P., Patrick, J.M., Wilson, A.J. Jr, 1977. Acute toxicity to and bioaccumulation of endosulfan by estuarine animals. In: Mayer, F.L., Hamelink, J.L. (Eds.), *Aquatic Toxicology and Hazard Evaluation*, pp. 241–252 ASTM STP 634: ASTM.
- Scott, G.I., Fulton, M.H., Crosby, M., Key, P.B., Daugomah, J.W., Waldren, J., Strozier, E.S., Loudon, C., Chandler, G.T., Bidleman, T.F., Jackson, K., Hampton, T., Huffman, T., Schulz, A., Bradford, M., 1992. Agricultural insecticide runoff effects on estuarine organisms; correlating laboratory and field toxicity test, ecophysiology bioassays and ecotoxicological biomonitoring (Final Report). U.S. EPA, Gulf Breeze, FL.
- Scott, G.I., Fulton, M.H., Moore, D.W., Wirth, E.F., Chandler, G.T., Key, P.B., Daugomah, J.W., Strozier, E.D., DeVane, J., Clark, J.R., Lewis, M.A., Finley, D.B., Ellenberg, W., Karnaky, K.J. Jr, 1999. Assessment of risk reduction strategies for the management of agricultural nonpoint source pesticide runoff in estuarine ecosystems. *Toxicol. Ind. Health* 15, 200–213.
- Scott, G.I., Fulton, M.H., Daugomah, J.D., Key, P.B., Leight, A.K., Wirth, E.F., Bearden, D.W., Pennington, P.L., Sumner, E.R., Chandler, G.T., van Dolah, R.F., 2000. Evaluation of Long-Term Monitoring of Grass Shrimp Population Life History as a Tool for Assessing Agricultural NPS Runoff Impacts. Presented at the 21st Annual Meeting for the Society of Environmental Chemistry and Toxicology, Nashville, TN, November 2000.
- Sikora, W.B., 1977. The Ecology Of *Palaemonetes pugio* In A Southeastern Salt Marsh Ecosystem With Particular Emphasis On Production And Trophic Relationships. University of South Carolina. Dissertation, 120.
- Soto, A.M., Chung, K.L., Sonnenschein, C., 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ. Health Perspect.* 102 (4), 380–383.
- Wirth, E.F., Lund, S.A., Fulton, M.H., Scott, G.I., 2001. Determination of acute mortality in adults and sublethal embryo responses of *Palaemonetes pugio* to endosulfan and methoprene exposure. *Aquat. Toxicol.* 53, 9–18.
- Wirth, E.F., 1999. Lipid and Reproductive Characterizations of the Grass Shrimp, *Palaemonetes pugio*, Exposed to Endosulfan or Methoprene. School of Public Health, University of South Carolina. Dissertation, 135.
- Wirth, E.F., Fulton, M.H., Chandler, G.T., Key, P.B., Scott, G.I., 1998. Toxicity of sediment associated PAHs to the estuarine crustaceans *Palaemonetes pugio* and *Amphiascus tenuiremis*. *Bull. Environ. Contam. Toxicol.* 61, 637–644.
- Zar, J., 1999. Comparing simple linear regression equations. In: *Biostatistical Analysis*, Third ed. Prentice-Hall, USA, pp. 360–376 Chapter 18.
- Zou, E., Fingerman, M., 1997. Synthetic estrogenic agents do not interfere with sex differentiation but do inhibit molting of the cladoceran *Daphnia magna*. *Bull. Environ. Contam. Toxicol.* 58, 596–602.